STUDIES ON THE VIRUS INOCULATION OF DISSOCIATED LEAF MESOPHYLL CELLS

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
Roger M. Knutson
1965

This is to certify that the

thesis entitled

STUDIES ON THE VIRUS INOCULATION
OF DISSOCIATED LEAF
MESOPHYLL CELLS

presented by

Roger M. Knutson

has been accepted towards fulfillment
of the requirements for

Ph. D. degree in Botany & Plant Pathology

Harry H. Mussakishi
Major professor

Date March 24, 1965

ABSTRACT

STUDIES ON THE VIRUS INOCULATION
OF DISSOCIATED LEAF MESOPHYLL CELLS

by Roger M. Knutson

The value of single-celled organisms in the study of cellular physiological processes is widely recognized. In the study of virus infection and biosynthesis, the phage-*E. coli* model has provided the basis for most proposed mechanisms. The ideal system for study of virus synthesis and related cell processes is a population of single, functional cells acting simultaneously. The study of higher plant virus infections at the cellular level has been inhibited by the necessity of using the leaf or portions of it as the unit of virus multiplication.

This study reports the infection of single, functional leaf-mesophyll cells. Such cells were obtained by the action of pectinase enzymes on strips of leaf tissue, and were maintained on a simple mineral nutrient medium plus sucrose for experimental periods. Cells were inoculated by the use of heat and violent agitation with an abrasive, and virus in excess of the quantity used as inoculum was detected in the cells by bioassay on a local lesion host plant.

Mesophyll cells infected with tobacco mosaic virus produced materials inhibitory to infection with that virus

on local lesion hosts. Use of acridine orange stains and fluorescent antibody precipitations was unsuccessful in determining specifically which cells were infected.

STUDIES ON THE VIRUS INOCULATION OF DISSOCIATED LEAF MESOPHYLL CELLS

By

Roger M. Knutson

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

1965

ACKNOWLEDGMENTS

Although a single name appears as author, many people have contributed, knowingly or unknowingly, to this work. Foremost among knowing contributors is Dr. H. H. Murakishi, whose help has been most welcome and most essential. The graduate students of the Department of Botany and Plant Pathology have provided most of the immediate environment in which I have worked for the past seven years. Their help and that of the total department faculty is more difficult to evaluate, but no less important.

DEDICATION

To my father, whose concern for the education of his children made it possible, this thesis is most sincerely dedicated.

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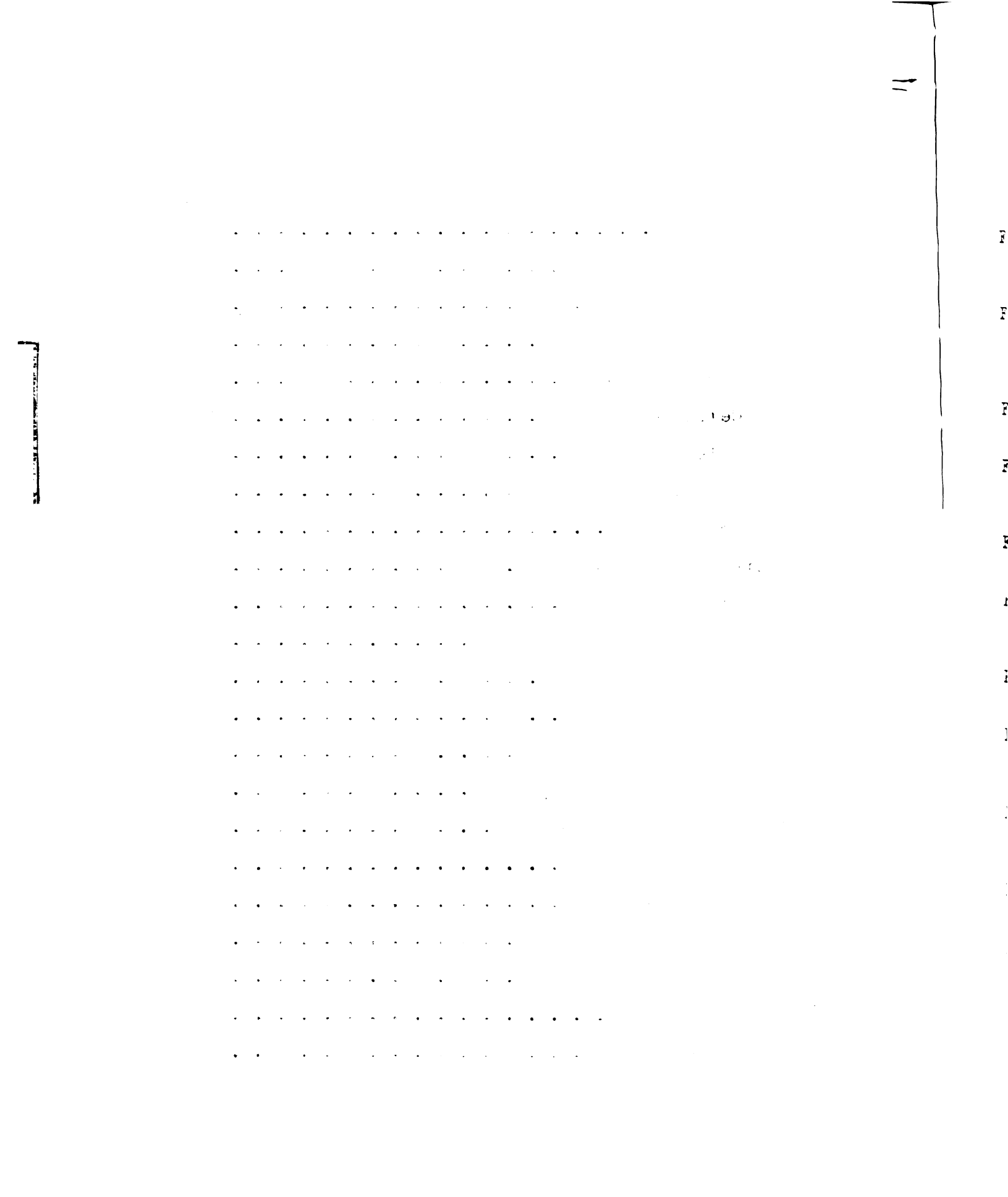

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INTRODUCTION

Plant viruses have been the subject of intensive investigation since early observations that certain maladies of plants could not be associated with any microbial agent. From the time of Stanley's and Bawden's crystallization of tobacco mosaic virus and its recognition as a definable chemical entity, investigators have been aware of the potential of virus-host systems in the elucidation of cellular physiological events. Understanding of higher plant virus infection at the cellular level has been slow in coming because of the difficulty of studying the infection process in single cells. Whole plants are the usual units, and there has been little change in the induction of virus infection since 1929 when Holmes demonstrated the efficacy of mild abrasion in enhancing infection. Today, as then, virus is rubbed on plant leaves with a variety of implements in such a way as to cause slight injury to the leaf. A small number of cells of the epidermis become infected, and the infection gradually spreads from cell to cell until most, if not all living cells of the leaf and eventually the plant, contain virus which they themselves have produced.

If the whole plant is considered as the infected unit, the process described above can demonstrate the effects of environmental and physiological changes on virus replication or symptom expression. The literature of virology contains

many references to the effects of temperature, light and other factors on virus quantity in the plant. Portions of the plant, leaves or leaf discs, have been successfully used in observations of the effects of various materials or conditions on virus synthesis or virus quantity. As the unit of study becomes smaller, it is increasingly less certain that it contains even one of the original infected cells of the leaf. Study of the very early stages of cellular infection is most difficult with systems using leaves or portions of leaves.

The functional unit of virus infection is the single cell. Furthermore, from what is known of virus synthesis, we can consider pathogenesis as a genetic rather than a metabolic phenomenon (Diener 1963). A virus particle enters a single cell and causes its own replication in that cell at the same time that infection is transferred to adjoining cells. The picture of virus infection at this level is that of one cell infected with a virus and producing its quota of new, complete virus particles. This does not occur in infected plants since adjoining cells become infected before the first cell has produced its full complement of virus. Study of cellular processes during virus infection must be in single cells going through one cycle of infection without being in organic contact with other cells. Outlined in full, the requirements of the ideal system for study of virus infection and replication at the cellular level are

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as follows. Single, physiologically functional cells of one kind must be available; they must be introduced to the virus simultaneously; and all or a known proportion must be infected. A single cycle of infection must occur, and virus produced must be effectively measurable. The similarity of the above requirements to the actuality of the phage-bacterial system is not accidental. The basic value of information obtained in many fields with the phage system is a testimonial to how closely it approaches the ideal.

I believe that an approach to such a system is possible with mechanically transmitted viruses of higher plants, and the work reported here is dedicated to that belief.

Single cells of higher plants are available by techniques of maceration or enzymatic digestion, and such cells have at least some of their in-place physiological activity. If leaf blades are used as the cell source, mostly mesophyll cells are obtained by enzymatic digestion. Single cells obtained from callus tissue cultures often demonstrate gross physiological and morphological differences. However, clones developed from single cells do not present these problems (Muir, Hildebrandt and Riker 1954, 1958).

Since higher plant viruses have no intrinsic means of bridging the cell wall, means of passing the wall or avoiding it are a necessary part of any technique. Simultaneous infection of cells can thus be relatively assured by the

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necessity of passing the cell wall.

The work presented here is not a study of the physiological events of early virus infection, nor is it concerned with the precise manner of virus entry into the cell, although studies of both might easily develop from it. The data and discussions involve only the demonstration that the infection of single isolated plant cells by viruses is possible, and that some cells are able to produce virus in significant amounts while supported on simple artificial media.

LITERATURE REVIEW

Introduction. The experimental work reported here is concerned with infecting single plant cells with viruses and observing the effects of such infection. There are few reports of such work in the published literature, and so far as I am aware, they have all utilized cells from plant callus tissue cultures (Gautheret, 1959). Single cell research as a model system for the study of cellular physiology has great value. The elucidation of the reactions and primary products of photosynthesis was not simple using Chlorella and Scenedesmus (Bassham, 1962), but it would have been much more difficult if whole leaves of higher plants had been the experimental units. The present study of cellular virus replication is similarly complicated by the use of leaves or portions of leaves as the units of infection. White (1959) explicitly stated the point when he said, "The cell is to biology what the molecule is to chemistry, it is the lowest common denominator of all life." This work is an attempt to reduce the study of plant viruses to the level of that lowest common denominator.

Cell availability. The first requirement if single cells are to be the unit of study is some means of obtaining them. Free-floating callus cells from tissue culture are available with appropriate culture techniques (Steward et al.,

1964; Northcroft, 1951). Quantities of physiologically active cells have been produced by careful grinding of leaves (Racusen and Aronoff, 1953), although the particular cells observed lost photosynthetic capacity rapidly and seemed to be deficient in protein synthesizing ability.

The knowledge that pectic materials and calcium ions occupy much of the interlamellar volume in plant tissues has been available for a long time, as has the knowledge that certain fungi produced enzymes attacking these materials (Wood, 1960). The use of pectic enzymes of various sorts, mostly pectin polygalacturonase (Devel and Stutz, 1958), for the gentle separation of cells was a logical development from that knowledge. The earliest reference to the use of pectic enzymes for cell separation is by Emsweller and Stuart (1944) who macerated anther tissue with fungus culture filtrates to facilitate observation of meiotic figures. Later, other workers used similar method to hydrolyze root tips and other plant materials (Hohl, 1948; Chayen, 1949). Commercial preparations of pectinases from Rhizopus tritici became available about 1950, and Orgell (1955) used such commercial materials to prepare plant cuticle for studies of foliar absorption. Tamaoki et al. (1960) treated callus tissues from culture with pectinase to help eliminate polysaccaride substances from mitochondrial preparations.

Enzymatic separation of cells for the specific purpose of studying virus replication within them was first accom-

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plished by Zaitlin in 1959. He obtained whole, individual mesophyll cells from tobacco leaf tissue, and was able to demonstrate that such cells from tobacco mosaic virus (TMV) infected plants, upon incubation, incorporated C^{14} labelled glycine or leucine into virus protein. Under some circumstances tissues do not separate easily with pectinase treatment, and since ethylenediaminetetraacetic acid (EDTA) had been shown to be effective in cell separation (Letham, 1960), Zaitlin and Coltrin, (1964) used EDTA in combination with more highly purified enzymes to separate cells from more refractory tissues. Earlier electron microscope observations (Wood et al., 1952) of pectinase separated cell walls revealed that the fibrillar portion of the cell wall remained intact and without holes.

The viability of cells obtained by any of the above means is a problem. Racusen and Aronoff (1953) reported that free cells produced no protein while excised leaf material did. They also noted a drop of 80% in photosynthetic capacity of single cells after one hour. In contrast with this are other observations indicating that some cellular activities are continued for long periods of time in dissociated cells. Chayen (1949) observed no mitotic figures initially in root tip cells separated with pectinase enzymes, but after four days in ringer's solution, the cells had apparently regained mitotic capacity. As previously noted, tobacco cells obtained by Zaitlin incorporated labelled amino acids into complete TMV. There are

other observations of long continued protoplasmic streaming in cells as an indication of metabolic activity (Wildman et al., 1962; Hirai and Wildman, 1963). Previous work indicates then, that single cells may be viable under quite a variety of conditions. There is, furthermore, some indication from whole leaf studies that ability to synthesize virus is retained even after synthesis of normal protein is suspended (Takahashi, 1941).

Virus infection. The manner of virus entry into the cell is one of the questions of vital concern in the infection of single cells with plant viruses. When whole leaves or portions of leaves are inoculated with virus preparations, the most common assumption has been that virus enters the cells through small temporary wounds and remains inside when the wound healing process is completed. The cells so infected are epidermal cells covered with a layer of cutin which must be at least locally interrupted if virus is to enter living cells through the hypothesized wounds. The leaf hairs have been considered another potential site of entry, an idea lacking current, wide acceptance. Ectodesmata, protoplasmic strands extending through the cell walls but not through the cutin layer, are currently considered the most likely sites of attachment and entry (Franke, 1961; Mundry, 1963; Brant, 1964).

Virus apparently can penetrate neither the tonoplast or the plasmalemma of uninjured cells (Mundry, 1963), although there are reports of infection occurring in single

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leaf hair cells from the contact of cytoplasm with concentrated virus preparations (Zech, 1952). Tissue culture cells incubated with sterile TMV have become infected with and without artificial wounding (Kassanis et al., 1958; Wu et al., 1960; Bergmann, 1959). There is then the possibility of viruses establishing infection in individual cells without abrasion. There is some evidence for the occurrence of pinocytosis (the uptake of small discrete volumes of material by invagination of the cytoplasm) in plant cells (Jensen and McLaren, 1960). Pinocytosis could provide a means for virus entry into the cytoplasm by cell activity rather than by lodging in wounded areas. Virus can be introduced by wounding single cells as shown by Benda (1958) who inoculated single cells of leaf hairs by scratching the surface with a single grain of carborundum in the end of a fine glass rod.

Once virus has entered or been taken into a cell, a complex series of reactions begins, leading to the final synthesis of viral particles with a character determined by the nucleic acid introduced. Since the concern of this thesis is a single cycle of infection in one cell, the timing of initial events in that first cell is important. The available information has come from several kinds of experiments. The earliest and most obvious measurement was increase of virus concentration in the infected plant. Later work concerned changes in the character of the infectious unit as determined by reaction to ultraviolet irradiation or HCl. Finally, removal of the epidermis at various intervals

after inoculation has given information on virus movement into underlying cells or from underlying cells to the epidermis.

Whole plant assays at intervals after infection show a generally sigmoid increase in virus concentration. Such results are, however, the product of two processes; the increase in virus per cell, and the spread of virus from cell to cell (Harrison, 1956). These two processes are inseparable when the only information available is the total quantity of virus present at some interval after the time of inoculation.

Work reported on plant viruses in tissue culture deals mostly with callus or crown gall tissue grown from virus infected plants (Kassanis, 1957; Hildebrandt, 1958). There have been few attempts to infect previously healthy tissue cultures with TMV (Hildebrandt, 1958; Augier de Montgremier et al., 1948; Kassanis et al., 1958; Bergmann, 1959). Several papers (Kassanis et al., 1958; Bergmann, 1959) report the infection of cell suspensions in liquid media. The incubation period used is long, and simultaneous infection was not attempted. However, subculturing at various intervals after inoculation, and subsequent assay of progeny may give information on the time of virus entry and percentage of cells infected. Studies of animal cells present no problem of one cycle of infection since they, like bacteria, release the mature virus into the medium (Ackerman, 1954). Kassanis et al. (1958) seem to suggest that such release of

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virus occurs in callus tissue infected with TMV, but their evidence is not conclusive, and there is no other report of such a phenomenon.

Post-inoculation changes in the response of virus particles to altered environments have provided almost the only information on early events following inoculation of plants. Treatment with ribonuclease during the first hour decreases the number of infections with TMV, while later treatments have no effect. (Bawden and Harrison, 1955). Two hours after inoculation, the virus is less readily inactivated by ultraviolet irradiation. At about four hours the UV inactivation curve departs from a first order slope, indicating the presence of more than one infectious unit per cell (Bawden and Harrison, 1955); a result confirmed for TMV by Siegel and Wildman (1956). After six hours, UV irradiation cannot prevent the further development of infection, indicating spread of infectious units to cells below the epidermis. The process suggested by these experiments is one of rapid attachment to a temporary site on the cell surface, penetration into the cell, loss of protein covering, multiplication in the cell and spread to adjacent cells (Harrison, 1959). While results are indirect, and the effect of UV or RNAase on the cells themselves is not evaluated, movement into adjacent cells at six hours is probably the most reliable measurement. Semal (1962) reports a period of six to eight hours during which TMV in-

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fection sites on Nicotiana glutinosa are susceptible to inactivation by treatment with 0.1 N HCl, indicating a change in state of the infected cell or site at about that time.

Since the large proportion of uninfected to infected cells early in disease development is the major problem in determining virus increase in leaf tissue, any reduction in this proportion would be valuable in obtaining information on virus increase in single cells. Dykstra (1962) and Fry and Matthews (1963) have accomplished such a reduction by using the attached epidermis as the infectible unit. By their calculation, this gives about an eight-fold increase in the proportion of initially infected cells. Migration of infection into the mesophyll occurs at about four hours after inoculation, and whole virus is first noted in the epidermal cells at seven hours. Tobacco mosaic virus-ribose nucleic acid (TMV-RNA) was used as inoculum to eliminate the confusion of residual virus on but not in the cells. This work provides the first good estimates of the time after inoculation during which the plant cell produces infectious units and whole virus. It also gives strong indications that the cell to cell movement of infection in the leaf is by RNA rather than whole virus.

Much slower movement of virus is revealed by the system of Hirai and Wildman (1963). They detected no increase in the infectivity of epidermal strips before the

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third day following inoculation. Residual virus could have masked early increases, since intact TMV was their inoculum for the underside of stem epidermal peels. Hair cells removed from epidermal peels showed no increase in infectivity prior to four days, which would indicate very slow movement of virus compared to the results obtained by Fry and Matthews. Nuclei in hair cells were observed to move toward the base or infected area at three days indicating some activity in these cells by 72 hours after virus was applied.

Cellular Activity. Activities of infected cells or infected plants have been investigated by numerous workers. Experiments involving direct observation of infected cells, whether live or stained, and investigation of metabolic activity or products have provided a basis for much speculation on the mode of virus synthesis. While direct cellular observations present numerous interpretive problems, and metabolic investigation of tissues is subject to errors introduced by the variety of cell types and states, some information so obtained has relevance to the study of single cell infections.

The laboratory of J. G. Bald has been the source of most recent cytological investigations of the virus infected plant cell. Stains for RNA and intact virus have been developed or modified (Bald, 1949, 1949a), and observations of live cells with phase microscopy have been correlated with the results of staining procedures (Bald,

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1964). The greatest difficulty with such work involves obtaining cells in early stages of infection when virus is being actively synthesized. Cells are obtained from tobacco plants three to five days after inoculation (Bald, 1964) and there is no certainty that the cells observed have not been infected for some time. Bald and others have made unquestioned observations of the early formation of crystalline inclusion bodies and the so-called X-bodies (Solberg and Bald, 1962), but relating nucleolar and nuclear observations to stages in virus synthesis is difficult (Bald and Solberg, 1961). Cell RNA and virus RNA cannot be differentiated by staining, and the effect of virus presence in the cell on cellular RNA synthesis or on cell ageing is not clearly understood. Since Fry and Matthews (1963) report virus synthesis within six hours after inoculation and Zech (1952) has observed what is interpreted as RNA synthesis within 30 minutes of virus introduction, the timing of cytological observations is critical. Positive correlation of cytological observations with timed events in early virus synthesis would make such observations extremely valuable.

The detection of virus within the cell requires methods more specific than the usual cytological stains. The preparation of flourescent dyes which can be attached to globulin protein gives such specificity for some virus components. Such dyes flouresce in the visible light range when irradiated with ultraviolet of an appropriate

wave length, and can be detected in very small quantities by microscopic observation. The labelling dyes can be quickly and simply conjugated with serum containing antibodies specific for almost any virus (Rinderknecht, 1962). Early techniques developed with animal cells and their viruses allowed investigators to count infected cells in tissue cultures (Deibel and Hotchin, 1959). Successful flourescent antibody staining has also been reported for pectinase-separated plant mesophyll cells (Nagaraj, 1962). The autoflourescence of plant cell walls and chlorophyll reduce the value of the method somewhat as compared to use with animal tissues, but it should be useful under proper circumstances.

Experimental measurement of respiration in virus infected tissues has been accomplished by many workers. A recent review (Millerd and Scott, 1962) summarizes most of the available information on respiration of virus infected tissues. Increases and/or decreases in overall respiration have been detected, depending on condition of plants, time after infection and numerous other unknown factors. Owen (1955) reported increases in respiration of tobacco leaves within an hour of inoculation with TMV, but he could not correlate the increase with any other manifestation of infection. Most such work has only emphasized that plant to plant variation is great enough to obscure any virus induced change (Merritt 1960). Since both wet and dry weight of infected tissue changes with

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time after infection, expression of respiration measurements compared to a healthy control is difficult. The energy for virus synthesis must come from increased metabolic activity, but the accurate measurement of that activity is difficult.

Specific and non-specific inhibitors of virus infection are among the products of virus infected cells. Ross (1961, 1961a) reported systemic induced resistance to virus infection, and considered the possibility that the resistance was due to the movement of some inhibitory material. The reaction described may or may not be the result of non-specific tissue necrosis (Bozarth et al., 1962; Bozarth and Ross, 1964). Loebenstein (1962,1963) produced a similar resistance to virus infection by inoculating plants with native virus protein, and later (Loebenstein and Ross, 1963) succeeded in isolating an inhibitory material from uninfected tissues on a plant with local lesions. The material is non-specific in that it inhibits infection by several viruses. Yarwood (1953) also described inhibition of infection on hypersensitive hosts, and found inhibitory substances in extracts from non-lesioned tissues. Sela and Applebaum (1962) discovered and later (Sela et al., 1964) partially purified a substance containing both protein and RNA, with inhibitory properties similar to those described by others. With the experimental systems used, it is impossible to determine whether the inhibitory materials are the product of in-

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The problems and areas of research discussed cover a large part of the whole field of present day plant virology. If there is any unifying principle it is that a system allowing the simultaneous infection of single cells would be helpful in the study of many aspects of virus infection at the cellular level.

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MATERIALS AND METHODS

Since many of the experiments performed here were concerned with development and evaluation of what is basically a method, only general information relating to the experiments as a whole, will be presented in this section. Materials and methods as they relate to the specific experiments will be presented and discussed with the results.

Origin of Materials. The virus used for most experiments was a rib grass strain of tobacco mosaic virus (obtained by Dr. H. H. Murakishi from Dr. F. O. Holmes in 1958) known hereafter as HRG. The virus has been maintained primarily in Nicotiana tobaccum var. White Burley. When purified virus was needed it was prepared from extracts of infected White Burley plants by the common methods of alternate low (10,000G) and high (93,000G) speed centrifugation. When plant extracts were used directly as a source of inoculum, the leaves were frozen, ground, strained through cheesecloth, centrifuged at low speed and appropriately diluted. Tobacco Necrosis Virus (TNV) used in several experiments was obtained from Dr. William Hooker. It was increased in primary leaves of bean and stored frozen until used.

Pinto bean plants used for bioassay of virus concentration were grown in a greenhouse without strict tempera-

ture control. Most frequently, seeds were planted in a 1:1 sand-peat mixture at a rate of two seeds per three-inch pot, although occasionally five or six plants were grown in larger pots. Whenever possible inoculations were made ten days after planting, although developmental stage rather than precise age of the plants was used as a guide. Plants with the primary leaves well expanded and with the first trifoliate leaves still folded seem most susceptible under our conditions. Seasonal variation in rate of growth was noticeable, but plants were never inoculated prior to nine days or after twelve days. Plants were selected for uniformity just prior to inoculation.

Assay Method. The plants used for most assays were Phaseolus vulgaris Var. Pinto. Variation in response of individual plants of the same variety has been observed, but the ease and speed with which large numbers of bean plants can be grown more than compensates for this difficulty. Since only the opposite primary leaves are used, four nearly identical half leaves are available for inoculation and comparison on each plant.

A variety of implements have been effectively used for inoculation. The experience of the operator would seem to be more important than the apparatus. In most of the work reported here, pieces of polyurethane foam approximately 1" X 1" X ½" were used to distribute the inoculum on the leaves. Leaves were not damaged, even by repeated rubbing, and the distribution of the resulting lesions was

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uniform over the leaf surface. The pads were washed and steam sterilized between uses. Since repeated rubbing of the leaf surface caused no macroscopically visible injury, an experiment was done to determine the relationship of lesion numbers to degree of abrasion. The number of times any one point on the leaf surface is rubbed should be the only important variable since pressure is largely controlled by the consistency of the foam pad.

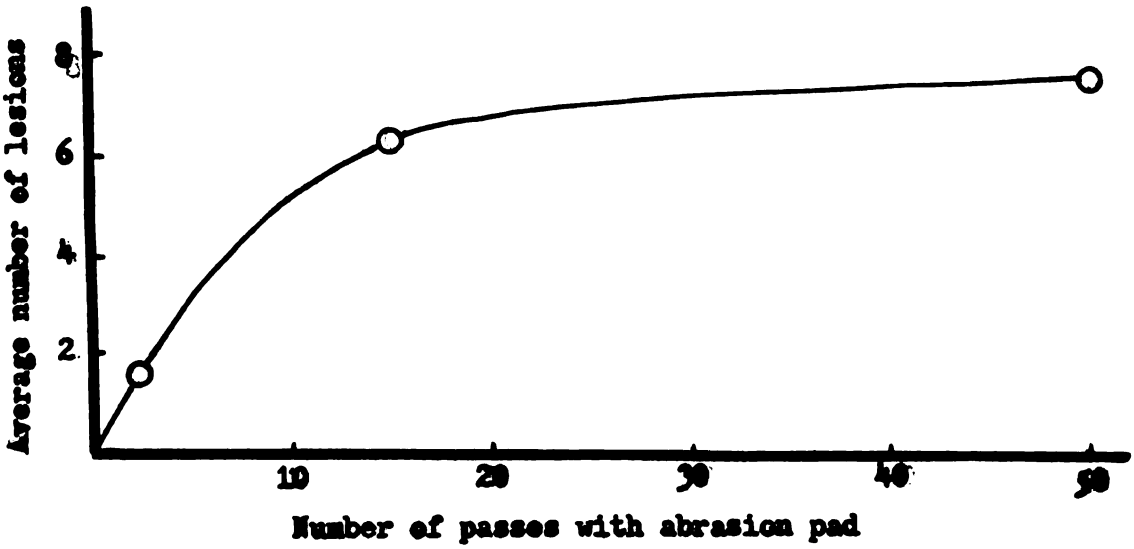

Fig.1.-Relationship of degree of abrasion to lesion numbers produced by rubbing with polyurethane pads. Twelve half-leaves per determination.

Since lesion numbers were not increased significantly ($P = .05$) (Bailey 1959) by abrasion beyond 15 passes, covering the leaf about three times over, 10-15 rubs, depending on the size of the leaf, were used for all determinations.

In order that results of experiments might be compared more directly, the same arrangement of assay samples was

maintained whenever possible. Each sample to be tested appeared on one half-leaf of six different plants, with one check half-leaf on each plant. Lesion numbers were totalled for the experimental and check inoculations on the same plants, and results expressed as percent of control. Lesions were counted at six to seven days since all lesions do not develop simultaneously and few lesions develop on Pinto bean after six days (Peacitelli and Santilli, 1961).

Several experiments were conducted to assure that the assay method would give reliable results. Bailey (1959) is the source of all statistical formulae used in the following evaluation.

The experimental design was such that the check appeared most frequently on the right half of the leaf, and since the operator is imperfectly bilaterally symmetrical, error could result from more abrasion on one half than on the other. Accordingly, an experiment was performed with 32 plants: 16 with the check on the right half and 16 with the check on the left. Means of the two sets are essentially identical, with no significant difference at the $P = .01$. Although these results indicate that location in the four possible positions on the assay plant has no effect on lesion numbers, a further test was designed with ten plants. All 40 half leaves were carefully inoculated with the same concentration of a purified virus preparation. Lesions were read and tabulated for

analysis of variance. As might be expected, there were significant (P = .05) differences between plants, but even if the most disparate within-plant differences were selected and six such plants compared, the differences between half leaves on the same plant were not significant. The variance from sources other than between plant and between half-leaves was high in all cases; a not unexpected result considering the known sources of variability in the local lesion bioassay for plant viruses.

As a final check on the validity of the assay method, three dilutions of purified virus were made at 1-500, 1-1000 and 1-2000 from an original concentration determined to be 5 mg/ml by Takahashi's (1951) spectrophotometric method. Pinto bean plants were inoculated with these dilutions; each dilution appearing on every plant. Using the 1-1000 dilution as a base, the results expressed as proportion of that dilution are 1.84:1.0:0.58, which results are not significantly different from 2:1:0.5. Bawden (1964) has indicated that quantitative statements about amount of virus must be made with great caution if lesion numbers are the only quantitative information available. Accordingly, most relative infectivity information is presented in graphs showing trends and general relationships rather than tables giving precise numbers.

OBSERVATIONS AND RESULTS

Cell preparation. The method used for preparation of leaf mesophyll cells was essentially that of Zaitlin (1959), modified somewhat for use with plants other than tobacco. Zaitlin cut leaves in narrow strips and shook them in a solution of .35 M (12%) sucrose, with .01% Pectinase (Nutritional Biochemicals Co.) and 1/15 M Sorensen's buffer pH 6-6.5. This concentration of sucrose works well for tobacco leaves, but best separation of cells from tomato leaves was obtained at about .23 M (8%) sucrose. Tomato mesophyll cells are generally larger than tobacco cells and more easily plasmolyzed, as determined by the effects of various sucrose concentrations on strips of leaf observed microscopically. Young succulent leaf tissue provides better yields of cells more quickly, and indeed, fully hardened tissues frequently do not separate noticeably even in double the usual pectinase concentration. The addition of Ethylenediaminetetraacetate (EDTA) (.001 M) as used by Zaitlin and Coltrin (1964) has proven beneficial for somewhat hardened tissues. Cells obtained by Zaitlin's method or modifications of it were washed repeatedly by centrifugation in .18 M (6%) sucrose-buffer, pH 7, and used as starting material for further experiments.

Preliminary Experiments. Simple sucrose-buffer and White's Solution with and without ferric citrate were used

as media in preliminary attempts to bring about detectable virus infection and replication. The virus (HRG) was added to the cell-medium mixture, which was centrifuged and resuspended four times. Cells were harvested at 72 hours, frozen, ground and centrifuged. A trace of antigen was demonstrated by microprecipitin tests (Ball, 1961), in the buffer soluble contents of cells maintained in sucrose-buffer. Various modifications were made, using microscopic observation of cells and micropricipitin tests as evaluation methods.

Since Yarwood (1961) had demonstrated that short periods of heating to 45-55°C. increased infections with TMV on local lesion hosts, and increase in temperature could be expected to accelerate most cell processes, various periods of heating were added to the inoculation procedure. The results of heating are shown in Fig. 2. Without heating, virus equal to 10% of control had been recovered; with heating, levels above 100% of control were reached for the first time, indicating that replication had definitely occurred. Since results are from a single experiment, the only conclusion drawn is that heating, even for short periods of time, increases virus recovery and ten minutes at 45°C. apparently does not kill the cells.

Results from variation of centrifugal force applied during the inoculation procedure are shown in Fig. 3. The cells were centrifuged twice at each indicated G force, incubated in Vickery's solution and sucrose for

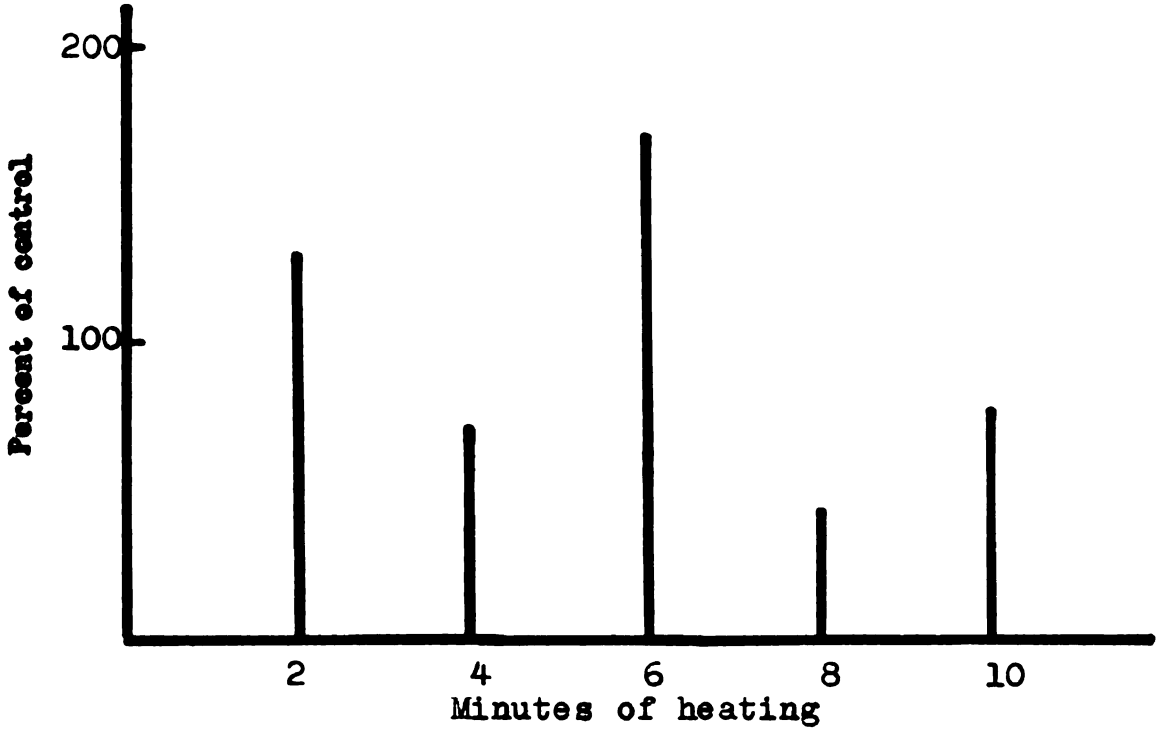

Fig. 2.-Effect of various periods of heating at 45°C. during the inoculation procedure on quantity of virus recovered at 40 hours. Results from a single experiment.

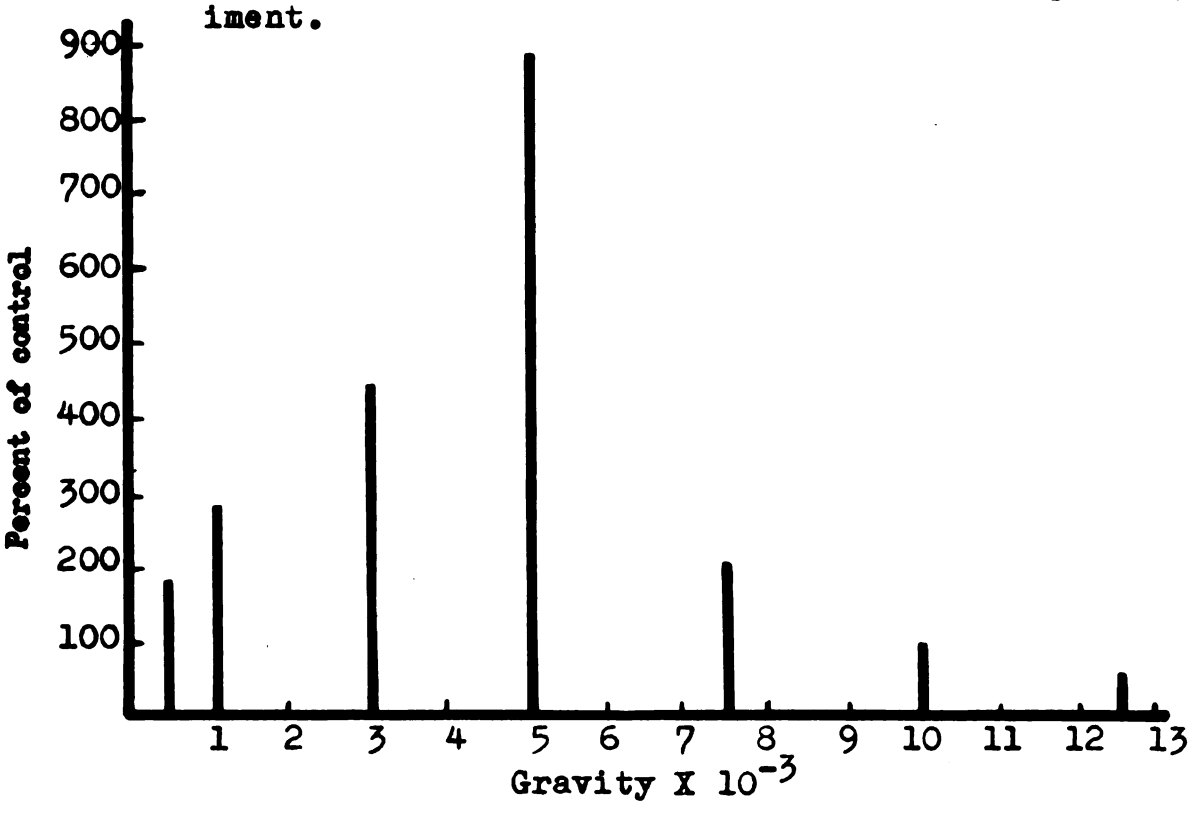

Fig. 3.-Use of the centrifuge as a means of inoculation. Level of virus measured at 40 hours. Results from two experiments.

40 hours, harvested and assayed on bean plants. The control for these experiments was a purified virus suspension, centrifuged, diluted and incubated without cells.

Even though virus was recovered in excess of the amount of inoculum when centrifugation was used as part of the inoculation procedure, damage to cells is probable at 3000-5000G. Agitation on a Vortex mixer (Scientific Industries, Inc.) was the most effective alternative method of abrasion tried. Cells are rapidly agitated in the presence of an abrasive and the virus, then incubated, harvested, and assayed as before. Figure 4 shows the results of one experiment with vortex mixing and celite as the abrasive. Timing of agitation is controlled precisely, and with nearly uniform amounts of celite and cells, the degree of abrasion should be reproducible.

Measurements with the Warburg respirometer indicate relative activity of the cells in several media tested, and show that respiratory activity of separated cells is much lower than that of very roughly equivalent amounts of leaf disc material (Fig. 5). Vickery's solution (KH_2PO_4, 0.143 gm/l; $CaCl_2$, 0.233 gm/l; $MgSO_4 \cdot 7H_2O$, 0.875 gm/l; $(NH_4)_2SO_4$. 0.555 gm/l) (Vickery *et al.*, 1937) has long been used to maintain leaves and parts of leaves for extended periods. It is a simple medium with known, chemically defined constituents, and it provided higher virus recovery than other materials tested in preliminary trials. Vickery's solution plus .15 molar sucrose was used as incubation medium

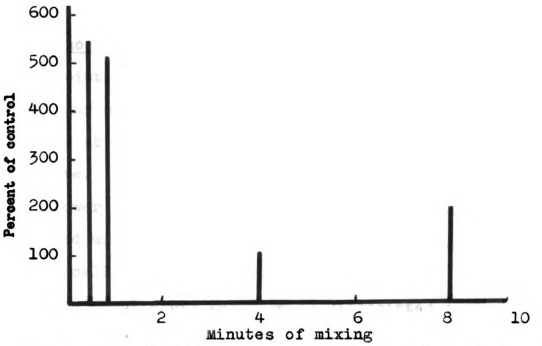

Fig. 4.-Effect of time of mixing with vortex mixer and celite in the medium on virus recovered at 40 hours. Results from a single experiment.

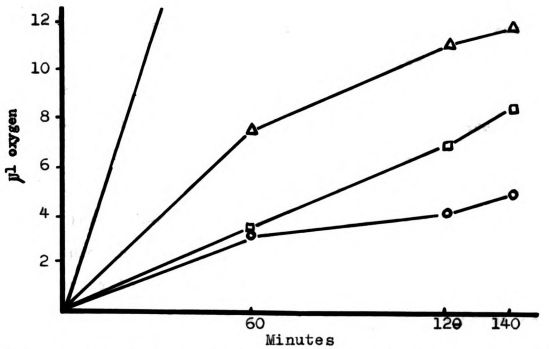

Fig. 5.-Respiration of dissociated cells in various media. O-White's mineral medium; □-Vickery's solution plus sucrose; △-Medium prepared from the solution in which cells were separated;—-tomato leaf discs.

in all following experiments.

Inoculation method. The inoculation method used at this point included addition of virus-containing clarified plant sap or purified virus to the cell medium mixture, heating at 45°C., and vortex mixing with celite as the abrasive. Carborundum was evaluated as an abrasive, but gave lower virus recovery than celite, and microscopically appeared much too large and dangerous compared to the size of the cells it was to abrade.

The respiratory response of dissociated cells to various steps in the inoculation procedure is shown in Fig. 6. On the basis of this data, agitation with celite increases respiratory activity of cells, at least temporarily. On the basis of preliminary data presented in Figs. 2,4,5 and 6, and other observations, the following procedure was adopted for most succeeding experiments. Washed cells were suspended in a minimum of medium, and mixed with purified virus suspension. The resulting solution was heated 8-10 minutes at 45°C., celite was added, and the whole mixture was agitated on the vortex mixer for 30 seconds. Aliquots of this inoculated cell suspension were pipetted into previously prepared 45 ml quantities of Vickery's solution plus sucrose in 125 ml flasks. The flasks were placed on a reciprocating shaker at a speed sufficient to cause some formation of bubbles in the medium, and were maintained in the dark at approximately 22°C for varying periods of time. The cells were then harvested by centrifugation at 3500G,

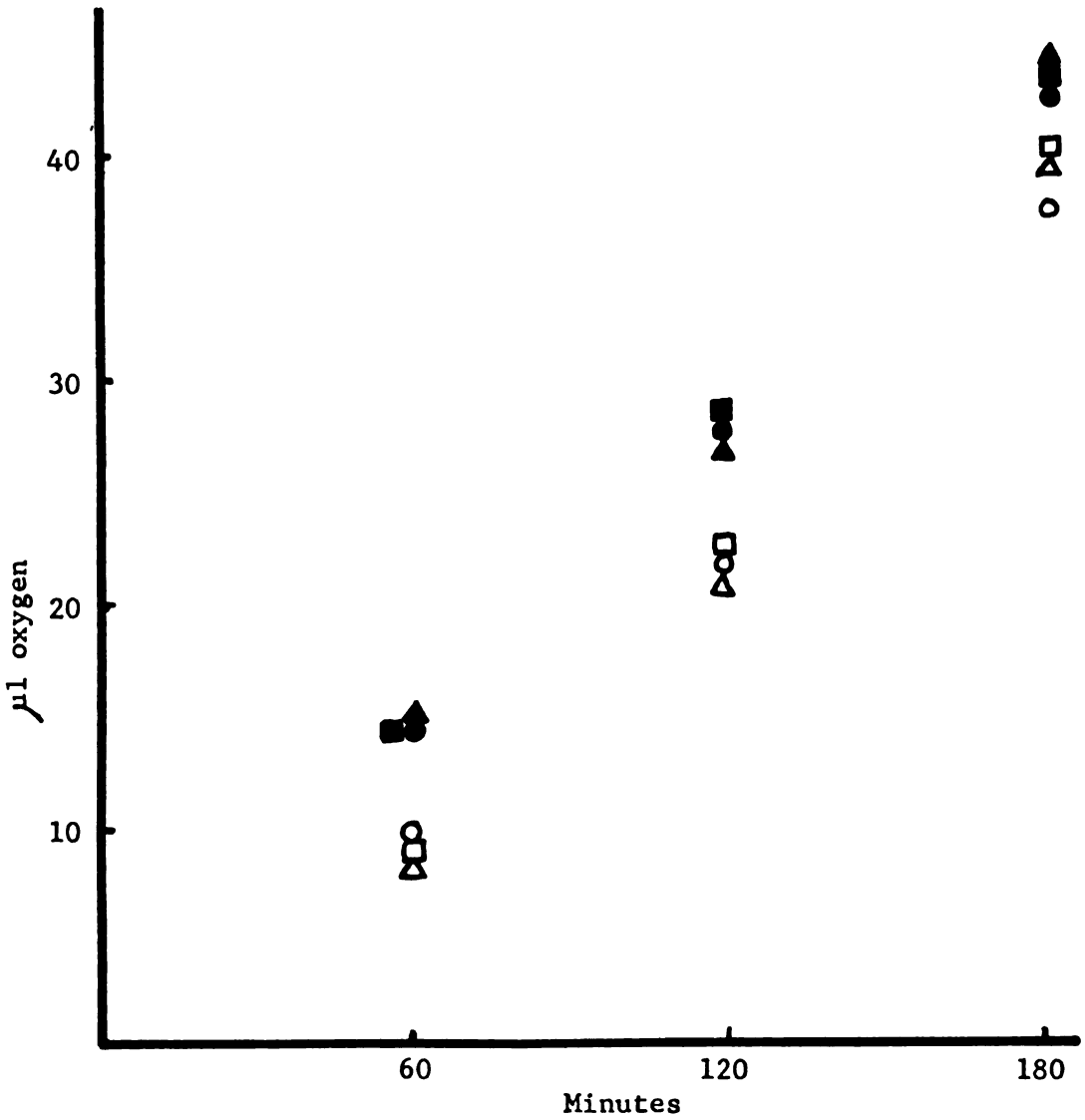

Fig. 6.-Respiration of dissociated tomato leaf mesophyll cells in various stages of the inoculation procedure. ○=Vickery's solution plus sucrose (V+S); △=V+S plus ten minutes heating; □=V+S plus heat plus vortex mixer (30 sec.); ●=V+S plus heat plus vortex plus celite; ▲=V+S plus heat plus vortex plus celite plus virus; ■=V+S plus heat plus vortex (two minutes) plus celite.

after washed, frozen and stored, as was a sample of the incubation medium. Just prior to assay, cells were thawed, ground in buffer and centrifuged. The supernatant of this centrifugation was the material indicated as buffer soluble cell contents and used to inoculate assay plants.

The presence in the medium of virus in excess of that actually entering cells, renders interpretation of results more difficult. Washing cells immediately after inoculation, before incubation, provides the simplest solution. Cells washed in four changes of medium immediately after inoculation contained small amounts of virus after 24 hours, as did the medium in which they were incubated. Such cells are injured by repeated washing after abrasion.

There are two major objections to leaving excess virus in the medium, aside from the difficulty of detecting early virus synthesis. First, the possibility that virus might become passively attached to the dying cells and give false indications of virus in the cells, and secondly that infection may occur later without the necessity of further heat or abrasion, giving erroneous ideas of the time required for virus synthesis. Attempts were made to eliminate these two latter possibilities.

Cells from healthy tomato plants were killed by freezing and thawing, then run through the usual inoculation procedure and incubated. Figure 7 shows results of virus assays of incubation medium and buffer soluble cell

Fig. 7.-Virus recovered from cells frozen and thawed before inoculation. Each point is an average value from two experiments. △=virus from medium; ○=virus from cells.

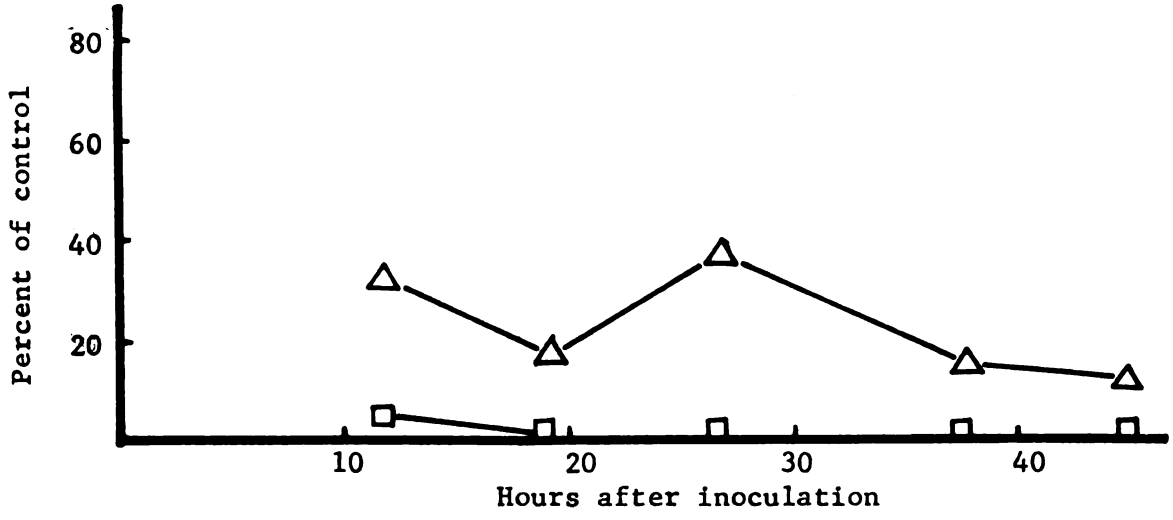

Fig. 8.-Recovery of virus from cells inoculated with and without heat and vortex mixing. △=with heat and mixing; □=without heat and mixing. Results from a single experiment.

contents of these dead cells at various times after inoculation. Virus associated with buffer soluble cell contents remains essentially constant for the total experimental period (30 hours), while virus in the incubation medium declines. If virus were becoming attached to or associated with non-living cell contents, increase of virus associated with cell contents should correlate with decrease of virus in the medium.

In another experiment, healthy cells were inoculated either with or without heat and vortex mixing. Figure 8 shows the results of this experiment. Without heat and mixing, buffer soluble cell contents contained practically no infective virus. Further indication that virus is not passively absorbed by dead cells, cell walls or the celite left in the medium is given by the fact that only traces of virus have ever been detected in the cell debris including celite, left after grinding and centrifuging the cell samples prior to assay.

Passive absorption of virus by cells is also discounted by experiments involving variation in numbers of virus particles and numbers of cells. If virus particles are being absorbed or passively taken into cells without replication, the amount of virus taken in and later assayed should be directly proportional to the number of cells in the medium. However, if multiplication is occurring, there should be an optimum number of cells for the amount of medium used, at which cell concentration the recovery

of virus would be greatest. Assuming that one virus particle is needed for an infection, at a constant cell concentration the virus recovered should be directly proportional to the amount of virus used as inoculum, up to some saturation point.

Cells are inoculated as before, but in one case cell numbers in the inoculation mixture are varied while virus concentration is kept constant, and in the other case, cell numbers are kept constant and virus concentration is varied. The results (Fig. 9) indicate that the optimum cell concentration is not the maximum used, and that recovered virus is proportional to the amount used as inoculum over the range of cell concentrations tested.

Another potential solution to the problem of residual virus is the use of virus RNA as the inoculum. Several attempts to inoculate cells with TMV-RNA prepared by the heat-salt method (Lippincott, 1961) failed. Apparently the RNA in the medium with cells was rapidly inactivated, and thus could not be used as inoculum in the present system.

The above results suggest that 1) passive attachment of virus to cells and accidental infection after time of inoculation can be discounted, and 2) RNA is not a useable inoculum. The use of a control level of infectivity as a base line rather than a zero level is therefore advisable. The control level is that infec-

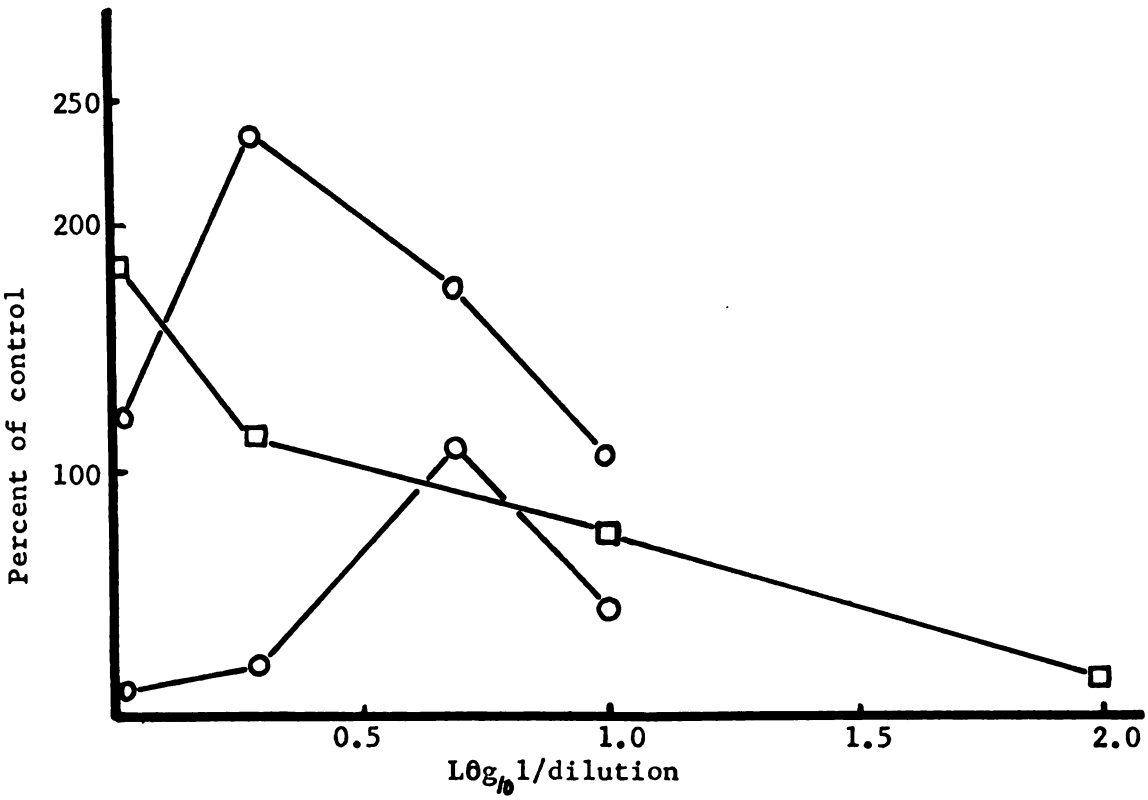

Fig. 9.-Separate effects of virus dilution and cell dilution on virus recovered at 24 hours. □=virus dilution; ○=cell dilution. Cell dilution data from two separate experiments.

tivity recovered from a dilution of purified virus equal to its dilution in the incubation medium.

Forty hours was selected as the period for virus replication on the basis of my early results with serological checks for virus presence. Later results showed that virus is produced in the cells in a much shorter time. However, the results at 40 hours may still have value. Since data Fig. 10 indicate that most virus outside the cells is rendered non-infective within 24-30 hours of the usual incubation, virus present at 40 hours after inoculation was probably produced by the cells after time 0. The information in figure 10 was gathered from numerous experiments in which infectivity above 100% of control was found in buffer soluble cell contents.

Curves showing loss of virus activity in cells separated from systemically infected plants also have some bearing on the detection of virus in cells 40 hours after inoculation. Results of several experiments with such previously infected cells are shown in Figure 11. Measured infectivity is low by 22-30 hours after inoculation with normal cell activity, but remains essentially unchanged when cell and microorganism activity is suppressed by merthiolate. Cells initially containing quantities of virus may be less viable than those without initial virus, but the difference is not likely to be great. Inactivation of the virus in the medium is likely caused by metabolic products of mesophyll cells or microorganisms. Virus

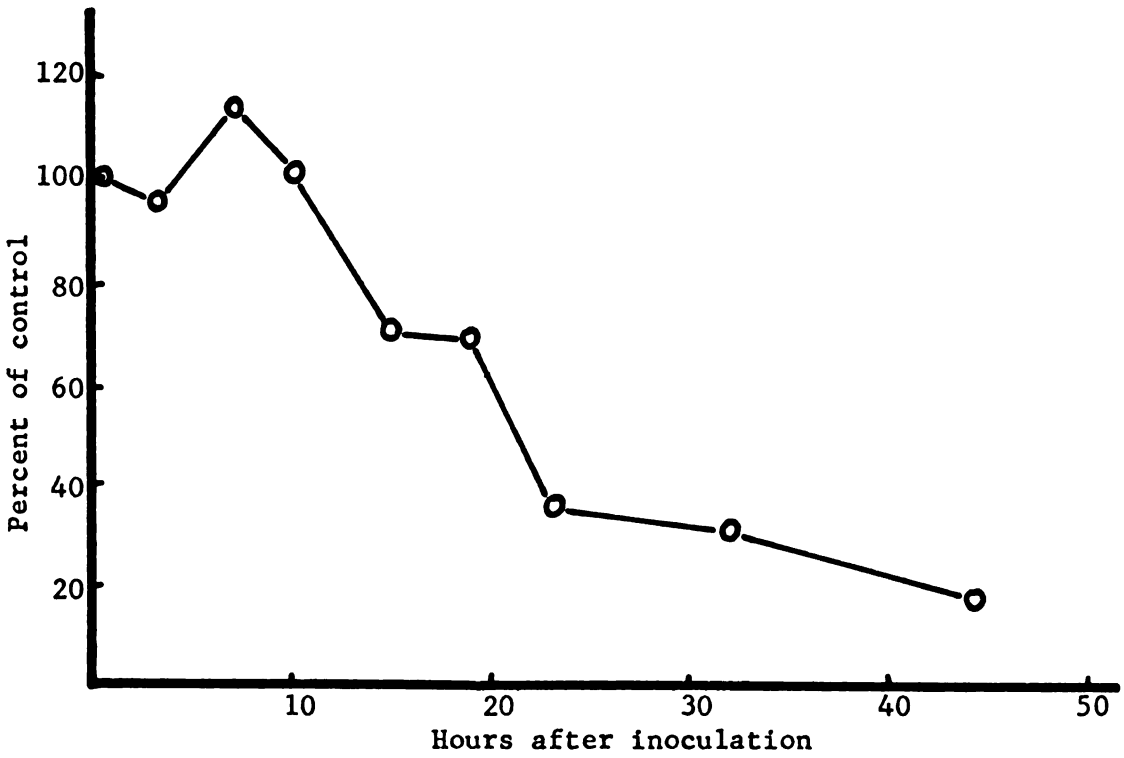

Fig. 10.-Loss of infectivity from medium supporting cells which produced virus. Each point is the average of from 5-17 determinations.

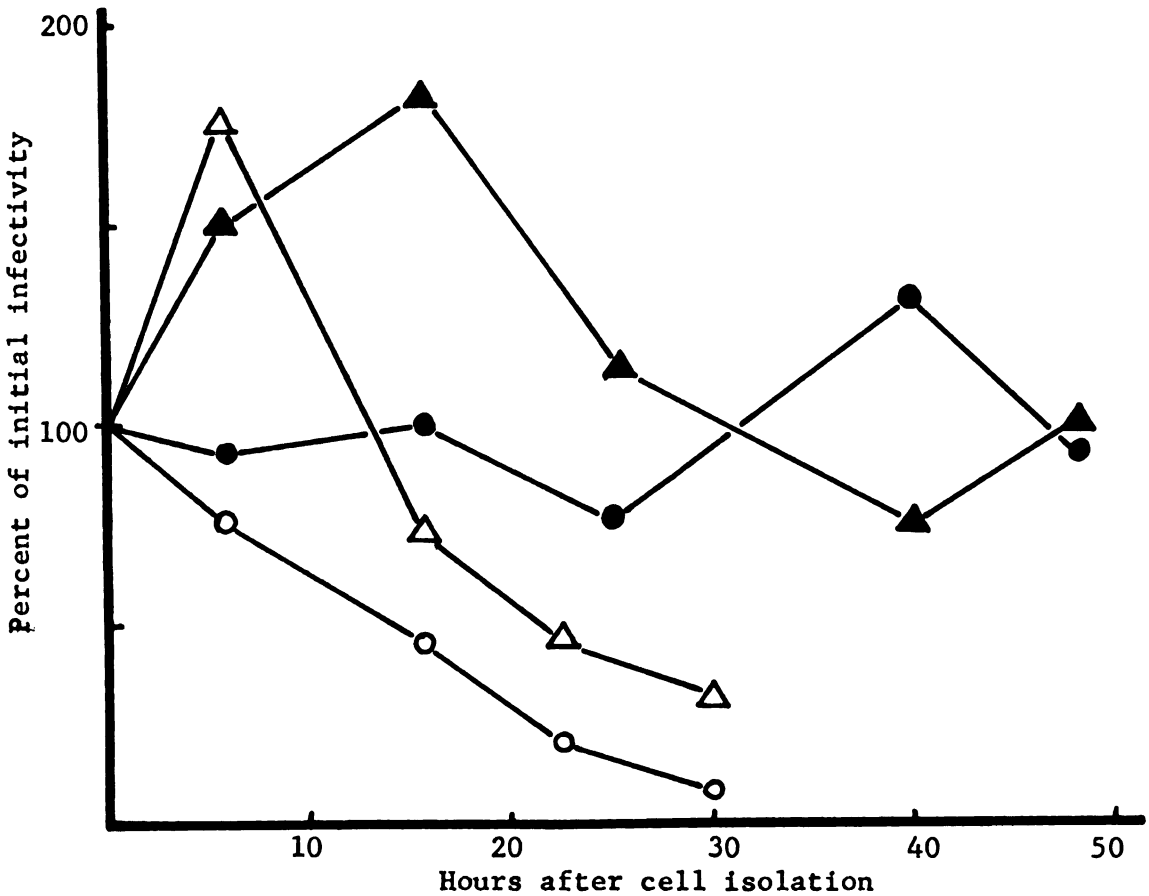

Fig. 11.-Loss of infectivity in cells isolated from systemically infected tomato leaves and incubated in vickery's solution plus sucrose. △=virus in medium; ○= virus in cells (average of two experiments). ▲=virus in medium with 10 ppm merthiolate; ●=virus in cells from that medium.

added as inoculum when merthiolate was not present, and remaining outside the cells would not survive much beyond 24 hours, and thus most virus found in the medium or associated with the cell contents at 40 hours must have been produced after time of inoculation.

The growth of microorganisms in the media presented problems for long term experiments, since the leaves from which cells were obtained could not be effectively sterilized. Several additives to the media were used in attempts to control microorganism growth. Experiments were done with merthiolate at one and ten parts per million in the usual Vickery's plus sucrose incubation medium. Virus quantities in inoculated cells never reached the check level, or reached it only after 30-40 hours when merthiolate was present. Respirometer measurements indicated 75% reduction in CO_2 exchange, and loss of virus from cells of systemically infected plants was negligible over a 48 hour period in merthiolate medium (Fig. 11). All the above would seem to indicate that while merthiolate at ten parts per million can effectively control microorganism growth for more than 100 hours, it also suppresses some cell activity when cells are exposed directly to it.

Penicillin and Chloromycetin (Parke-Davis Chloramphenicol) were also used in efforts to control bacterial contamination, but neither seemed effective in suppressing overall microorganism population in the medium.

Up to about 24 hours the growth of fungi and bacteria

is not sufficient to present problems of general competition or changes in the medium. After 30 hours medium pH dropped slightly and the mesophyll cells were probably affected. Barring some highly specific inhibitory interaction, the time period up to 24 hours should give results not seriously complicated by the presence of microorganisms.

Virus synthesis. Many experiments were performed in an attempt to determine the relationship of time after inoculation to virus quantity in the cells. As reported above, 40 hours was initially considered to be the earliest time at which measurable increases in virus could be demonstrated. Most frequently at this early stage of experimentation, no samples were taken prior to 12 hours after inoculation, and some results (Fig. 12) suggested a virus increase peak at about 20 hours.

As the processes of cell separation, inoculation and incubation were repeated, the speed and efficiency with which each step was accomplished increased. The result was cells in a healthier or more lively condition at any stage, even though no gross differences could be observed. Infectivity was significantly above that of the control at 12 hours, the earliest time at which samples were taken, but declined thereafter (Fig. 13). When samples were taken earlier than 12 hours, the peak was obtained at 7 hours, followed by a decline (Fig. 14). The data shown in Fig. 14 utilized an incubation medium other than Vickery's solution, but indications are that only quanti-

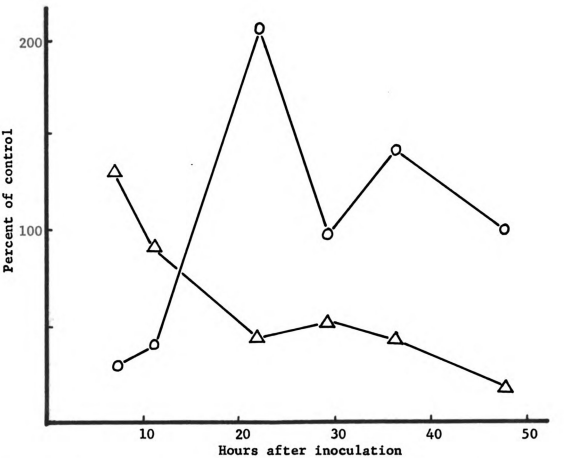

Fig. 12.-Increase of virus quantity with time. Each point is an average from two comparable experiments. △=virus from medium; ○=virus from cells.

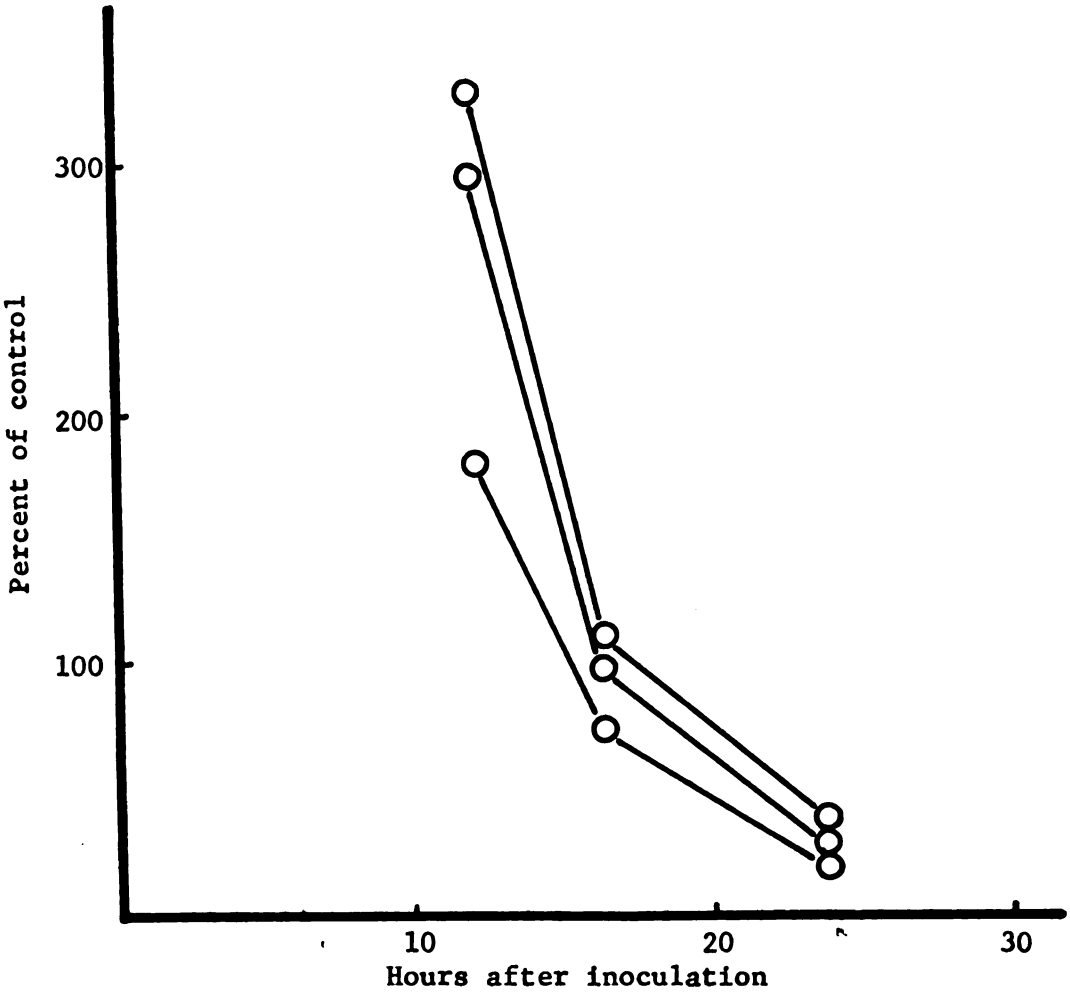

Fig. 13.-Results of three experiments showing decline in virus recovered from cells in the period from 12-24 hours after inoculation.

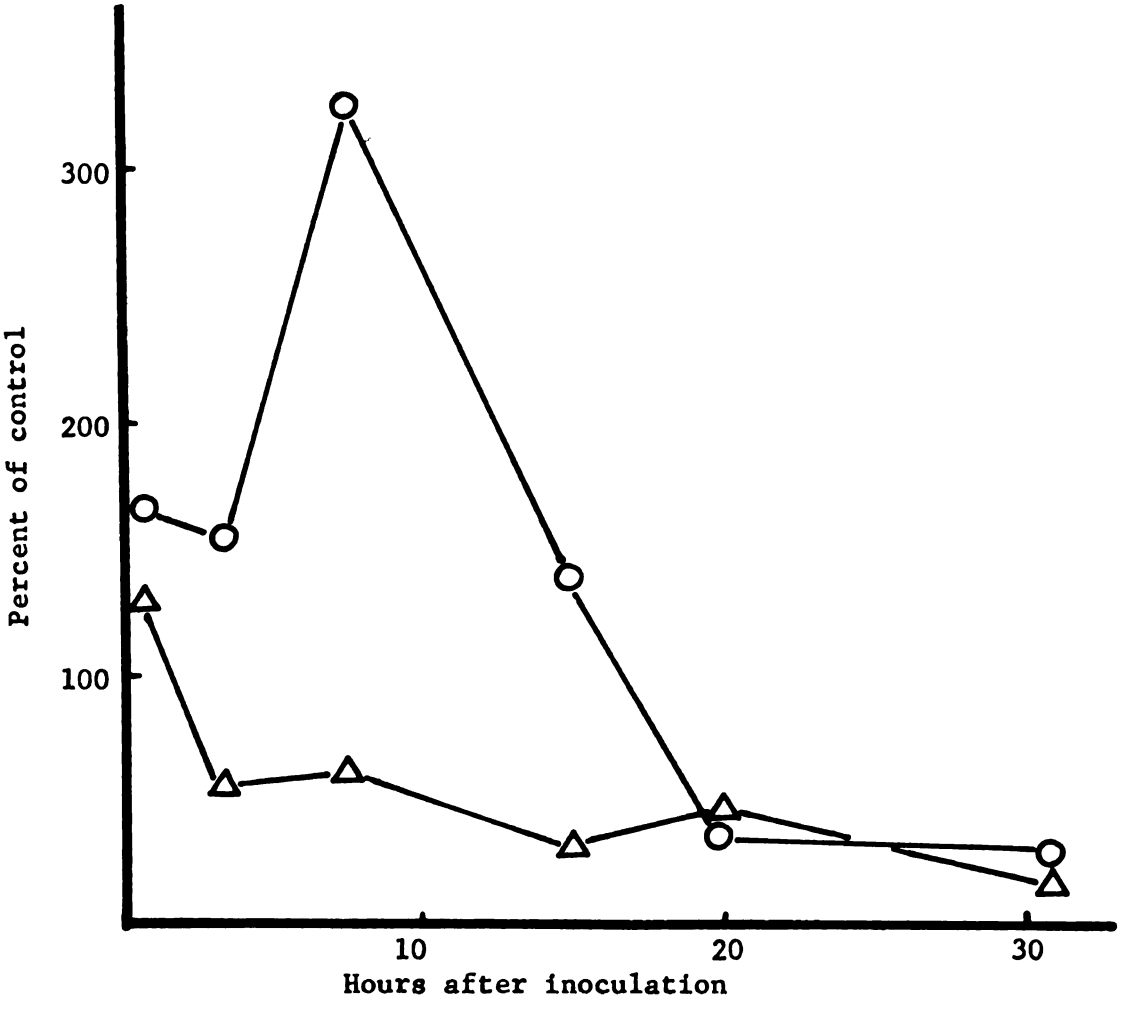

Fig. 14.-Results showing a peak in virus quantity at seven hours. △=virus in medium; ○=virus in cells. Each point is the result of two replications of the inoculation and incubation.

tative differences in cell response are involved.

For experiments with TMV then, the earliest peak of virus quantity observed was at seven hours, which is in agreement with observations of Fry and Matthews (1964), for the time required to replicate virus in cells functioning as part of a whole leaf. Similar early high values were obtained in several experiments with TNV, but the small number of trials and some uncertainty in the local lesion assay casts doubt on these results.

If the greatest virus quantities obtained from inoculated cells at times of seven hours and after are plotted on a time scale, the resulting line has the negative slope that would be expected for the measurement of any product of a population of organisms declining in overall health (Fig. 15). A decline in overall health is believed to be involved in the leaf mesophyll cells used in these experiments.

Cells were inoculated and assayed for TMV in 22 comparable experiments, and the results treated statistically (Fig. 16). The curved line represents the 5% significance level expressed as percent of control for the various mean values of the control half leaves. Any point above the curved line indicates that an amount of virus significantly larger than that used as inoculum was found in buffer soluble cell contents. Results of seventeen separate comparable experiments with cells from two plant genera indicate that intracellular virus

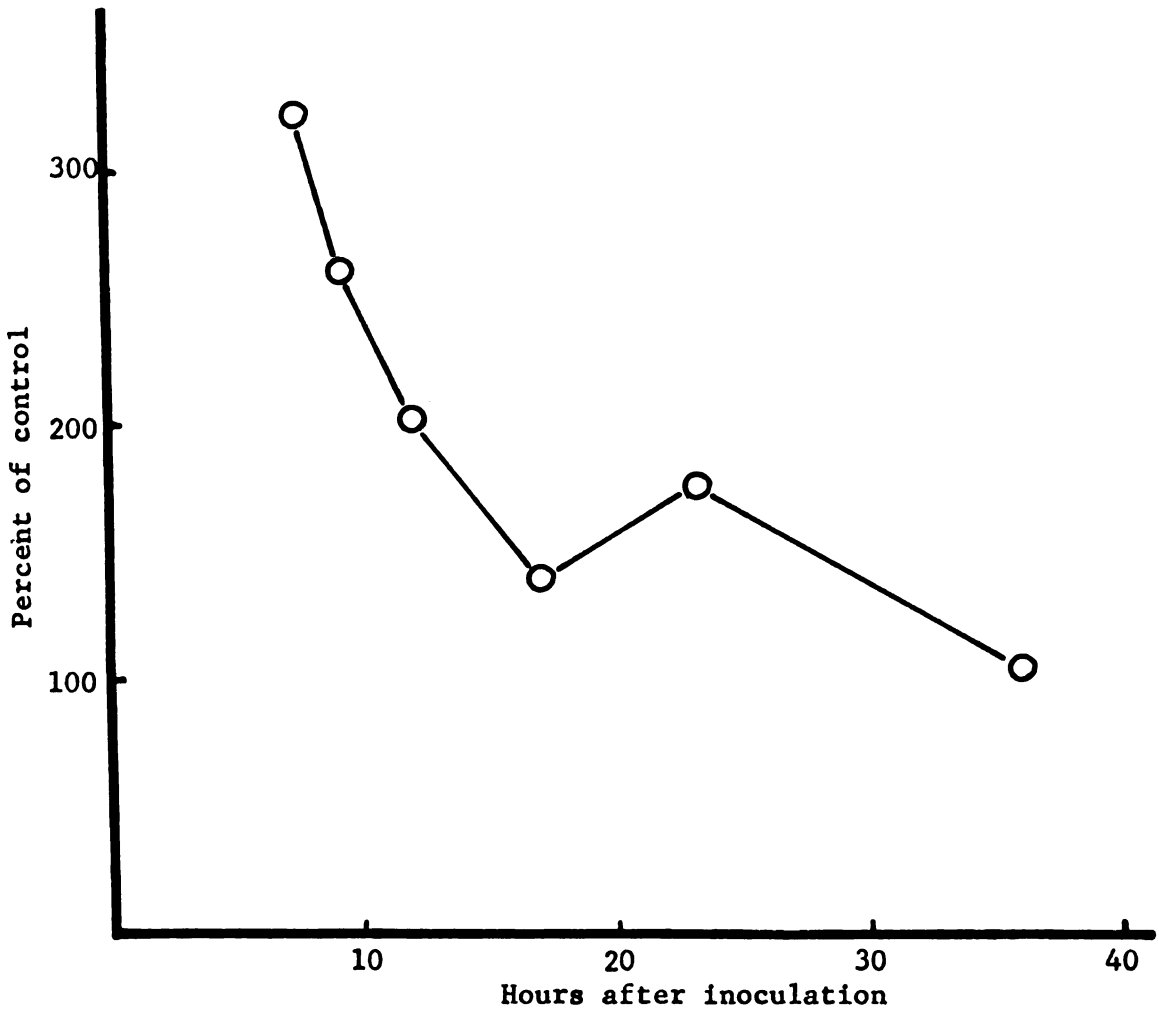

Fig. 15.-Plot of the high values obtained at each time interval after inoculation. The negative slope indicates loss of capacity to produce virus.

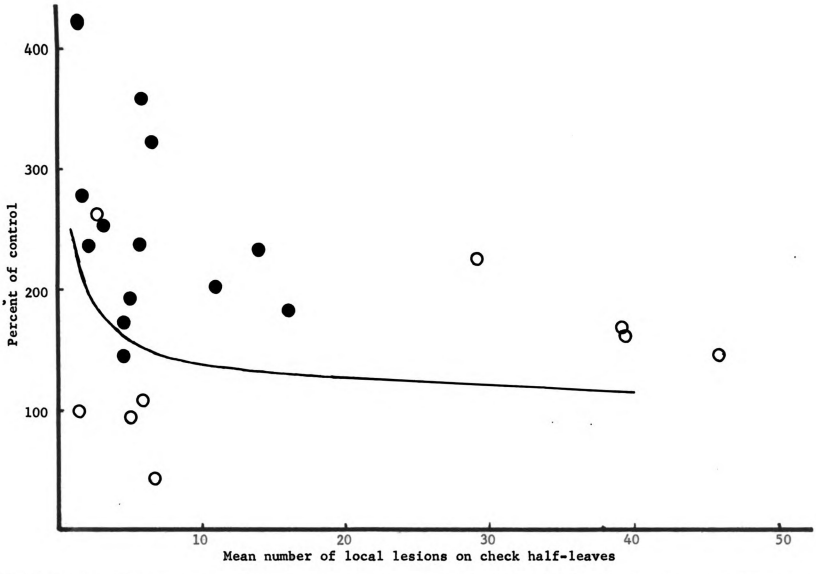

Fig. 16.-Plot of 22 comparable experiments in which cells were inoculated and later assayed for virus content. ●=tobacco cells; ○=tomato cells. Curved line indicates significant increase above 100% (P=.05)

multiplication occurred.

Inhibitor formation. Plants infected with viruses have been shown to produce extractable materials which are inhibitory to virus infection when mixed with the virus prior to inoculation. An experiment was therefore designed to determine if inhibitors were present in cells infected as individual units. Cells were inoculated in the usual way, and at 24-30 hours harvested, frozen, ground and treated with hydrated calcium phosphate (HCP) according to the method described by Sela and Applebaum (1962). Extracts containing potential inhibitory materials were prepared from tobacco cells and tomato cells, both inoculated and uninoculated, and from the medium in which cells had been incubated. Purified virus preparations were diluted with the solutions so prepared, and the resulting inoculum was applied to Pinto bean, the usual local lesion assay plant. The control was a dilution of the virus in .01 M phosphate buffer, pH 7. Results from two experiments with tomato cells are shown in Table 1. Tobacco cells produce inhibitory materials that tend to obscure the difference between inoculated and non-inoculated cells. These results support the observations (Zaitlin and Siegel, 1963) of a non-specific inhibitor from pectinase separated tobacco cells. No serious attempt was made to identify the inhibitory material or materials. Without infection and intracellular virus production it is unlikely there would be a difference in the amount of

inhibitor between inoculated and uninoculated cells. Inhibitory materials were produced in the presence of the virus in greater amounts than were found in uninoculated cells.

Table 1.-Inhibition of infection by cell and culture fluid extracts. Percent inhibition is calculated with the buffer diluted purified virus as the base. Results from two experiments.

Material	Percent inhibition	
Medium alone	12	4
HCP treated		
Frozen ground cells		
Inoculated	50	56
Uninoculated	0	20
Incubation medium		
Inoculated	67	23
Uninoculated	11	0

Infected cell detection. The proportion of infected and non-infected cells in any experiment would be valuable information; however no difference could be observed between inoculated and non-inoculated cells by direct microscopic observation. Several means of demonstrating differences were therefore attempted.

Flourescent antibody stains were prepared from rabbit antiserum to the ribgrass strain of TMV (HRG) and flouresceinisothiocyanate by the method of Rinderkneckt (1962). This is a simple,rapid method utilizing a column of "Sephadex" for separation of dye-protein conjugate and unconjugated dye. Cells from infected plants and healthy plants were

treated with the prepared flourescent antibody and observed with a Zeiss flourescence microscope, using the method outlined by Nagaraj (1962). Flouresence was observed within cells from infected plants, but also within some cells from healthy plants. No filter system used could eliminate the flouresence from all healthy cells and retain it in all infected cells. Isolated inoculated cells compared to isolated healthy cells had similar patterns of flouresence. Although Nagaraj reported he could distinguish cells from healthy and TMV infected plants by this method, in the present study the condition of any one particular cell seems impossible to determine with his method or several variations of it. Probably only a small percentage of cells are infected by the inoculation method described here, and without some means of determining the state of virtually every cell, information from flourescent antibody staining is not conclusive.

A method utilizing acridine orange stain on killed and fixed cells (Hooker and Sumanwar, 1964) gave similar conflicting results with dissociated healthy and diseased cells. It may well be that cells separated by pectinase digestion and maintained in artificial media are sufficiently different from cells in place on the leaf to invalidate methods demonstrated effective with leaf tissue.

The problem of what percent of cells can be infected by the method developed here remains unsolved, and is one of the critical problems for future work. Staining methods

may provide the answer, and would prove a much more rapid means of evaluating variations in the inoculation procedure; a task that is long and complicated when the amount of virus recovered at some later time is the only indication of success or failure.

DISCUSSION AND CONCLUSIONS

The work presented here proposes to answer only one question: Can individual mesophyll cells obtained from leaf tissue be infected with tobacco mosaic virus by the methods described? Most of the experiments and observations reported, excluding those concerned directly with methods, bear on this question.

Cell maintenance. The success achieved in maintaining cells on simple mineral media supplemented with sucrose indicates that for the short periods of time involved, mesophyll cells are able to carry on some complex activities requiring internal energy transformations. More precise means of evaluating the responses of cells to various media should make it possible to select more effective nutrient mixtures.

Cell inoculation. The inoculation procedure developed has the one outstanding quality of functionality, and analogies to the whole plant inoculation procedure are acknowledged. Improvements may require completely new approaches. Without more precise knowledge of the means by which plant viruses normally enter and infect cells, improvements may have to be based on further guesses as to what changes in treatment of cells will increase infection. Some stimulation of the cells seems necessary. Respiration measurements show increases in activity of

the cells after agitation with the abrasive Celite, (Fig. 6) and the measurements of recovered infectivity show the desirability of using heat as part of the stimulatory process. Whether the stimulated cell actively takes in portions of the liquid environment including virus particles, or whether the virus itself is involved in attachment and entry into the cytoplasm was not determined. Such questions may however, be more easily answered in a single cell inoculation system than in one using intact tissues.

Determination of the time period during which virus is produced in populations of single cells is subject to various errors in the system used. In the intact leaf virus RNA apparently travels to adjacent cells before whole virus is produced in the initially infected cell (Fry and Matthews, 1964). It is not known whether such movement out of the cell is still possible when the plasmodesmata are not connected to adjacent cells, although figures seen in tomato stem tissue (Livingstone, 1964) show plasmodesmata extending through the walls of separated cells. Unless one assumes nearly 100% initial infection, a quick buildup to a low carrying capacity and an accelerating cell mortality, the progressive loss of infectivity from the culture medium would argue against release of stable infectious material. Virus RNA leaving the cells would probably be inactivated quickly by ribonucleases released from broken cells (Frisch-Niggemeyer and Reddi, 1957), and even if not inactivated, the amount

would probably be too small to reflect in infectivity measurements considering the low efficiency of TMV-RNA as inoculum (Lippincott, 1961). A peak of virus quantity for the population of cells was observed in buffer soluble cell contents as early as seven hours after inoculation. Assuming the validity ofthe assay method, the peak must represent whole virus particles produced and retained in the cell during those seven hours. In other experiments peaks of activity were later, but since earliest values are highest (Fig. 15), seven hours can be taken as a figure approaching the minimum.

Inhibitor production. The experiments on inhibitor production were not concerned directly with measurement of infectivity, but rather with determining if cells were infected at all. The basic assumption was that two groups of cells treated identically except for the presence of virus would differ in metabolic activity. The virus may enter the cell and cause metabolic derangement resulting in production of virus components. If this does not occur, the plant cell continues normal function. If it does occur, the redirection of cell metabolism would result in the production of substances other than those directly required for virus synthesis.

A significant difference in inhibition was observed between materials associated with inoculated cells and those associated with uninoculated cells. Specific inhibitory materials differing from normal cell products may

have been produced by infected cells, but the experiments show only the amount of inhibition not its direct cause. Production of more or different inhibitory materials by inoculated cells is one of the best pieces of evidence that virus synthesis or some part of the infection process is occurring in inoculated cells.

Validity of results. All of the results expressed in terms of infectivity stand or fall on the validity of the assay method. This being the case, evaluation of the method is a necessary part of the work. Numerous trials indicated that the bean plants used were sufficiently uniform for effective half-leaf assays. Several other factors not precisely controlled are involved in the results obtained from assays. The direction of potential error in all cases is such as to reduce rather than increase difference between control and experimental results. Three primary factors are responsible for the uncontrolled variability: 1) production of inhibitory substances by infected cells, 2) use of dilutions of purified virus for controls, and 3) the loss from and inactivation of virus in infected cells.

Judging from the results of experiments demonstrating inhibitors, there is reason to suppose that all infected cells produce materials which inhibit the demonstrable infectivity of the virus they synthesize and contain. Such inhibitors would be expected to lower infectivity results and give indications of less virus than the cells had

actually produced. No attempt was made to study inhibitor production for periods less than 24 hours after infection.

The use of direct dilutions of purified virus as control inoculum would give accurate indications of change in infectivity of cell extracts only if we assume that no virus is inactivated in the incubation medium during the experimental period or during storage. The departure from accuracy is in the direction of less virus over and above that of the control rather than more. Kassanis et al. (1958) used virus added to aliquots of medium without cells and incubated for the same length of time as the control inoculum. Figure 4 shows an experiment indicating 875% of control virus level. This result was obtained with a control similar to that used by Kassanis, and may be contrasted with a high of 324% of control obtained using direct dilutions of purified virus as the control inoculum.

The loss of virus from cells with time is most clearly shown when they are isolated from systemically infected plants. With the culture system used, such cells show rapid loss of demonstrable infectivity during 24 hours. During that same period, cells isolated from healthy plants and inoculated with TMV showed rapid increase in contained infectivity. It is reasonable to suppose that the observed increase masked the loss of some virus from inoculated cells. A better indication of virus produced would be obtained by adding the observed loss to the observed gain and using this value for the capacity of

dissociated cells to synthesize virus.

The above factors combined with the normal loss of virus known to occur when infected plant material is macerated and centrifuged, allow the realistic supposition that virus synthesis did occur in the inoculated single cells, and may be higher than the measured values indicate.

It would be premature to state that the advantages of a single cell inoculation system as outlined in the introduction are available for the study of plant viruses. Progress has been made, however. Single cells with at least some synthetic capacity are available. Inoculation procedures that infect at least a portion of a cell population have been developed. And most importantly, much evidence indicates that those single cells actually produce whole, infective virus in measurable amounts. Means of maintaining sterility in the system would be helpful, but would present great technical problems without wholesale changes in procedure. A means of determining which cells in a mixture have been infected and are producing virus is high on the priority list for future work. In short some progress toward the ideal system outlined in the introduction has been made. Further effort should be rewarding.

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